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The Identification and Determination of Barbiturates in Serum

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The identification of barbiturates is based on the difference in the distribution coefficients of various barbiturates between chloroform and each of two aqueous alkaline solutions and, furthermore, on differences in the shapes of the absorption spectra for the enol forms of various barbiturates.
The identification and quantitative determination is performed by extracting the serum containing a barbiturate, at pH 7.4 with 50 times its volume of chloroform. Part of the chloroform layer is extracted with dilute NaOH and the absorption of the extract at 230, 240 and 250 mμ is measured at pH 10 and pH 2. Another portion of the chloroform layer is extracted with Na_2HPO_4 (pH 8.9). The type of barbiturate present is determined from the difference in absorption at 240 mμ of this extract at pH 8.9 and pH 2, together with the previous absorption differences at 240 and 250 mμ.
The concentration of the barbiturate in serum is calculated from the difference in absorption at 240 mμ of solutions at pH 10 and pH 2, using a reference curve for barbital. The value found in this way is multiplied by the appropiate barbiturate factor to give the correct concentration.
The confirmation of barbiturate poisoning is a qualitative as well as a quantitative problem. Before the onset of toxic symptoms, the blood levels of the long-acting barbiturates, such as phenobarbital (luminal) and barbital (veronal), are much higher than those of the short-acting ones, such as hexabarbital (N-methylcyclohexenyl methyl barbital = evipan). Barbiturates with medium action, such as butobarbital (soneryl), pentobarbital (nembutal) and allobarbital (dial) also show intermediate blood levels.

Die Auftrennung von Barbituraten beruht auf der Verschiedenheit der Verteilungskoeffizienten verschiedener Barbiturate zwischen Chloroform und zwei alkalisch-wäßrigen Lösungen, sowie auf den Unterschieden in der Form der Absorptionsspektren für die Enolformen verschiedener Barbiturate.
Die Identifizierung und quantitative Bestimmung wird durch Extraktion des Barbiturat enthaltenden Serums bei pH 7,4 mit dem 50fachen Volumen Chloroform der Ausgangsserummenge vorgenommen. Ein Teil der Chloroformschicht wird mit verdünnter NaOH ausgezogen und die Absorption des Extraktes bei 230, 240 und 250 mμ bei pH 10 und pH 2 gemessen. Ein zweiter Teil der Chloroformschicht wird mit Na_2HPO_4 (pH 8,9) extrahiert. Der anwesende Barbiturattyp wird aus der Differenz in der Absorption dieses Extraktes bei 240 mμ bei pH 8,9 und pH 2,0 — zusammen mit den obengenannten Absorptionsdifferenzen bei 240 und 250 mμ — berechnet.
Die Konzentration des Barbiturates im Serum wird aus der Absorptionsdifferenz bei 240 mμ der Lösungen bei pH 10 und pH 2 berechnet, wobei eine Bezugskurve für Barbitursäure verwendet wird. Der auf diese Weise gefundene Wert wird mit dem passenden Barbituratfaktor multipliziert und ergibt so die korrekte Konzentration.

SUNSHINE (1) reviewed the correlation between the concentrations of different barbiturates in blood and the established clinical condition. — Comatosis may appear at a blood concentration of 65 mg/*l* for a long-acting barbiturate, 45 mg/*l* for an intermediate-acting and 25 mg/*l* for a short-acting one. — To determine the seriousness of a barbiturate poisoning in a comatose state, it is — in addition to the determination of its concentration in serum — important to know, which barbiturate is present or to which group (long, medium, or short acting) it belongs. Generally, when the patient is not comatose, there will be little necessity to know which barbiturate was taken. Therefore, the identification of barbiturates in most cases will only be necessary if their concentrations in the serum are relatively high.

Several spectrophotometric methods for the quantitative determination and identification of barbiturates are described in the literature (2—8). Identification methods for barbiturates are described by STEVENSON (2), HELLDORF (5) and GOLDBAUM (4). VAN HAERINGEN (3) gave a review and a critical examination of several of these methods. For quantitative determination it appeared that the extraction of acidified serum with a large excess of chloroform and determination of the absorption at 240 mμ in a buffer solution of pH 10 and at pH 2 gave

the most satisfactory results. The method according to GOLDBAUM (4) gives an indication of the type of barbiturate, but does not identify it specifically.

Method

Barbiturates can exist in two tautomeric forms, viz, the enol-form and the keto-form, depending on the pH of the solution. The absorption maximum for barbiturates in weakly alkaline solutions (pH 8.9 and pH 10) is caused by the enol-form and appears at approximately 240 mμ, with slight variations for the various barbiturates. In acid solutions (pH 2) the barbiturates exist in the keto-form and show no absorption at 240 mμ. It must be supposed that the physical properties of a barbiturate, as well as the formation of its enol-or keto-form, at any given pH, are influenced by its substituents. Indeed, DYBING (7) reported that the distribution coefficients of various barbiturates between chloroform and an aqueous solution showed large differences at the same alkaline pH. He also indicated that the distribution coefficients for one barbiturate between chloroform and aquous solutions of different alkalinities may differ considerably.

The findings of DYBING are confirmed by our investigations. The identification of barbiturates, according to the method described below, is based on the difference in distribution coefficients of the various barbiturates between chloroform and two aqueous alkaline solutions and furthermore on the difference of the absorption curves for the enolforms of the various barbiturates.

Three sera, each containing 200 mg of a different barbiturate, were submitted to the identification and determination procedures described below. The sera were extracted with chloroform. One part of each solution was extracted with an aqueous solution, pH 8.9. The other part was extracted with 0.1 N NaOH to extract the maximum quantity of barbiturate. This alkaline solution was adjusted to pH 10 with boric acid to ensure that the barbiturates were present in the mono-enol form (7). Absorption difference curves between the aqueous solution of pH 10 and pH 2, as well as those of pH 8.9 and pH 2, for wavelengths between 225 and 260 mμ, are shown in figure 1.

Figure 1 shows that the absorption differences at 240 mμ between pH 8.9 and pH 2 and those between pH 10 and pH 2 show ratios that are quite different for each of the barbiturates, although their initial weight concentrations in serum are *equal*. The different ratios are explained by the different distribution coefficients of the barbiturates between the chloroform and the alkaline solutions. The identification of a barbiturate can be achieved on this basis, and by using the ratio of the differences in absorption at 240 and 250 mμ with solutions of pH 10 and pH 2. This ratio is more or less different for each of the barbiturates, and is caused by the different slopes of the absorbance difference curves between 240 and 260 mμ. From the determined absorption for each barbiturate, two parameters are composed that are placed as absis

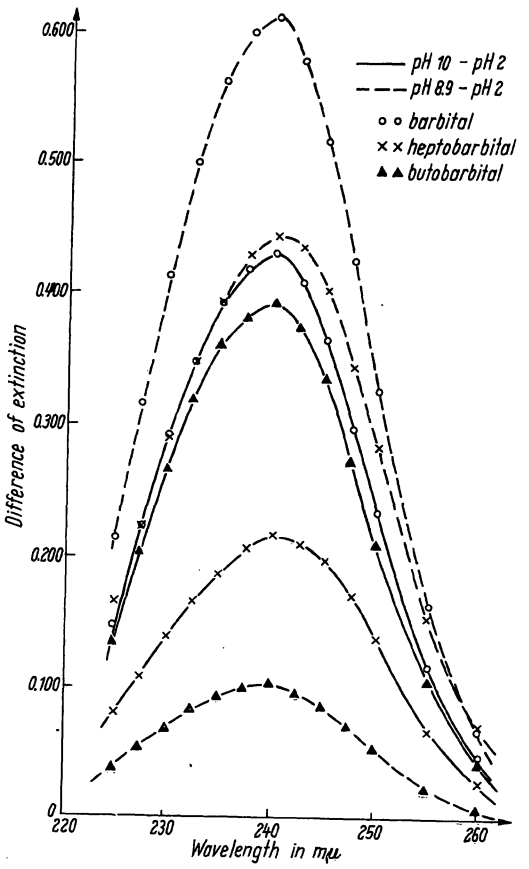

Fig. 1
Spectral absorption difference curves of barbiturates between aqueous solutions of pH 10 and pH 2 as well as those of pH 8.9 and pH 2, obtained from sera containing 200 mg/l barbiturate

and ordinate in a graph. *Parameter I*, defined as the ratio at 240 and 250 mμ of the absorption differences between pH 10 and pH 2, *is placed on the absis* in a graph.

$$\text{Parameter I} = \frac{A\ \substack{\text{pH 10—2}\\ 240\ \text{m}\mu}}{A\ \substack{\text{pH 10—2}\\ 250\ \text{m}\mu}}$$

On the ordinate of the graph *is placed parameter II*, which indicates the ratio of the absorption differences between solutions of pH 8.9 and pH 2, and those between solutions of pH 10 and pH 2 for 240 mμ.

$$\text{Parameter II} = \frac{A\ \substack{\text{pH 8.9—2}\\ 240\ \text{m}\mu}}{A\ \substack{\text{pH 10—2}\\ 240\ \text{m}\mu.}}$$

In this way every barbiturate has its own place in the graph (fig. 2), although there is some overlapping. The quantitative determination of a barbiturate in serum is performed according to a reliable method reported by VAN HAERINGEN (2), using the linear relation between the measured absorption differences at 240 mμ in the solutions of pH 10 and pH 2, and the barbiturate concentration in the serum. This relation is different for various barbiturates as shown in figures 3a and 3b. This method for quantitative determination and slight variations of it, have been described previously by WALKER c.s. (6), DYBING (7) and LOUS (8).

Identification and determination

Reagents

Phosphate buffer pH 7.4: 0.908 g KH_2PO_4 and 4.750 g $Na_2HPO_4 \cdot 2H_2O$ are dissolved in 100 m*l* distilled water.
Chloroform, redistilled, bp. 59—61°.
0.1 N NaOH (A.R.).
Boric acid solution: 6.2 g boric acid (A.R.) in 1 *l* 0.1 N KCl. A mixture of equal volumes of this solution and 0.1 N NaOH should form a buffer with pH 10 · 0 (test with indicator paper).
4 N HCl.
Phosphate solution of pH 8.9: 8.90 g $Na_2HPO_4 \cdot 2H_2O$ (A.R.) are dissolved in 250 m*l* distilled water.

Procedure

Extraction of sample with chloroform
Add to a 100 m*l* separating funnel, 1 m*l* serum, 0,4 m*l* phosphate buffer of pH 7.4 and 55 m*l* redistilled chloroform; shake for 5 minutes.
After separation, the chloroform solution is placed in a dry receiver, which is closed with a stopper and used for the extraction procedures at pH 8.9 and pH 10.

Procedure at pH 8.9
25 m*l* of the chloroform solution, in a 100 m*l* separating funnel, are extracted with 6 m*l* disodium hydrogen-ortho-phosphate solution (pH 8.9) by shaking for 5 minutes. After separation, the chloroform layer is removed and the ortho-phosphate solution is centrifuged for 5 minutes at 3000 rpm.
The absorption of the clear solution is determined with a spectrophotometer in a quartz cuvette at 240 mμ. 6 Drops of 4 N HCl are then added to the solution in the cuvette so that the pH becomes 2 or less (test with indicator paper) and the absorption is determined again at 240 mμ. The absorption at pH 2 is substracted from that at pH 8.9.

Procedure at pH 10
25 m*l* of the chloroform solution, in another 100 m*l* separating funnel, are extracted with 6 m*l* of 0.1 N NaOH by shaking for 5 minutes. The chloroform layer is removed and the NaOH solution is centrifuged for 5 minutes at 3000 rpm. 3.0 m*l* of the clear solution are transferred to a test tube with a pipette and mixed with 3.0 m*l* of boric acid solution. The mixture now has pH 10.0. The absorption of this solution is determined with a spectrophotometer in quartz cuvettes at 230, 240 and 250 mμ.
2 Drops of 4 N HCl are then added to the solutions in the cuvettes. Now the pH is 2 or less (test with indicator paper). The absorption is determined again at 230, 240 and 250 mμ. The values at pH 2 are substracted from those at pH 10.0. A barbiturate is present, if the absorption differences show a maximum at 240 mμ (see note 2).

Notes
If the absorption at 240 mμ and pH 2 for the "*procedure at pH 10*" is higher than 0.150 and for the "*procedure at pH 8.9*" is higher than 0.100, an interfering substance may be present. The chloroform solution should then be washed twice with 1 m*l* of 30% H_2SO_4, once with 1 m*l* of water and finally once with 0.5 m*l* of phosphate buffer, pH 7.4.
For prominal the maximum is found at 250 mμ; however the absorption difference at 240 mμ is definitely greater than that at 230 mμ.

Identification

The substance is plotted in the barbiturate diagram (fig. 2) by glacing the value of parameter I on the absis and that of parameter II on the ordinate. The determined position indicates the identity of the barbiturate present.

Determination of the concentration in the serum

The barbiturate concentration is determined from the absorption differences between pH 10 and pH 2 at 240 mμ, by comparison with those of a calibration curve for barbital (fig. 3). The determined concentration is multiplied by the appropiate barbiturate factor.

Example
Identification and determination of serum samples A and B, containing 100 mg/*l* each of phenobarbital and allobarbital, respectively.

pH of solution	Absorption at 230 mμ A	230 mμ B	240 mμ A	240 mμ B	250 mμ A	250 mμ B
8.9			.264	.201		
2			.051	.037		
difference			.213	.164		
10.0	.365	.306	.320	.288	.238	.206
2	.244	.204	.148	1.26	.124	.103
difference	.121	.102	.172	.162	.114	1.03

	Substance A	Substance B
Parameter I:	$\frac{.172}{.114} = 1.50$	$\frac{.162}{.103} = 1.58$
Parameter II:	$\frac{.213}{.172} = 1.24$	$\frac{.164}{.162} = 1.01$
Identification:	phenobarbital	allobarbital
Barbiturate factor:	1.3	1.2

Concentration in serum: $81 \times 1.3 = 105$ mg/*l* $76 \times 1.2 = 91$ mg/*l*

Discussion and Results

Identification

To identify a barbiturate in a case of poisoning we direct ourselves in first place to those substances that are used in a definite country or region and especially to those, that are easily available to the public. Table 1 shows a survey of the values of parameters I and II composed from ten determinations for each of the various barbiturates.
Figure 2 shows the places of the substances mentioned in table 1, in a barbiturate graph. The places are obtained by placing in the graph for each of the barbiturates the mean values of parameters I and II plus or minus twice their standard deviations. This means that 95% of all possible plots for a barbiturate in the diagram are covered by the areas of the indicated place.
The long-acting barbital, phenobarbital and heptobarbital are found in the higher part of the diagram. Methylphenobarbital, a N-methyl barbital, which is also a long-acting barbiturate, however is found in the lower part of the diagram. Of the examined barbiturates, heptobarbital (rutonal), methyl phenobarbital (prominal), hexabarbital (evipan), barbital (veronal), phenobarbital (luminal) and allobarbital can be identified. Butobarbital (soneryl), heptabarbital (medomin) and pentobarbital (nembutal) cannot be distinguished from each other.

Evaluation of concentration

For various barbiturates, the absorption differences at 240 mμ between solutions of pH 10 and pH 2, are different for equal weight concentrations as shown in table 2.

Tab. 1
Parameters I, II and barbiturate factors for several barbiturates

Barbiturate		Mol. weight	Parameter I Mean*	Parameter I Stand. dev.*	Parameter II Mean*	Parameter II Stand. dev.*	Barbiturate factor
Methylphenobarbital	(prominal)	246	0.91	0.047	0.17	0.120	2.1
Hexabarbital	(evipan)	234	1.11	0.049	0.12	0.065	1.8
Pentobarbital-Na	(nembutal)	248	1.70	0.050	0.20	0.090	1.3
Heptabarbital	(medomin)	250	1.80	0.067	0.28	0.060	1.3
Butobarbital	(soneryl)	212	1.86	0.080	0.30	0.045	1.0
Cyclobarbital-Ca	(phanodorm)	275	1.62	0.062	0.44	0.048	1.1
Aprobarbital		210	1.53	0.034	0.56	0.055	1.2
Allobarbital	(dial)	208	1.53	0.048	0.96	0.058	1.2
Phenobarbital	(luminal)	232	1.52	0.070	1.15	0.060	1.3
Barbital	(veronal)	184	1.86	0.113	1.36	0.083	1.0
Heptobarbital	(rutonal)	218	1.52	0.042	1.90	0.180	1.8

* Calculated from 10 determinations for each of the barbiturates

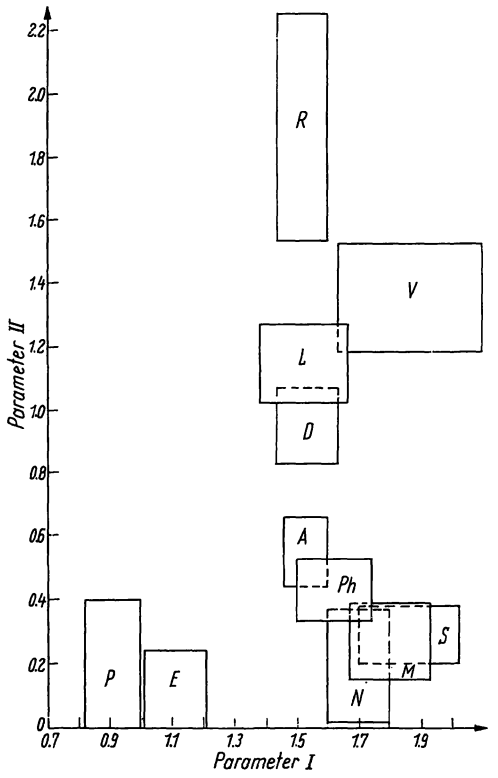

Fig. 2
Barbiturate diagram.
Places of barbiturate expressed in parameters I and II

P	= methyl phenobarbital	(prominal)	A	= aprobarbital	
E	= hexabarbital	(evipan)	D	= allobarbital	(dial)
N	= pentobarbital	(nembutal)	L	= phenobarbital	(luminal)
M	= heptabarbital	(medomin)	V	= barbital	(veronal)
S	= butobarbital	(soneryl)	R	= heptobarbital	(rutonal)
Ph	= cyclobarbital	(phanodorm)			

Figures 3a and 3b show the graphical representation of the data mentioned in table 2.
It is evident that for the determination of the barbiturate concentration in serum we have to use the corresponding calibration line for the barbiturate that is present. In practice it will be more convenient to use only the calibration curve for barbital and to multiply the determined concentration with a factor corresponding to the barbiturate that is present. This barbiturate factor indicates the weight concentration ratio for equal absorbances between a barbiturate and barbital.

A survey of the barbiturate factors for several generally known barbiturate obtained from figure 3a, b is given in table 1. The barbiturate factors correspond rather well with the molar ratios of the barbiturates with respect to barbital, except methyl phenobarbital, hexabarbital, heptobarbital and cyclobarbital.
The mean absorption difference for 18 sera without barbiturate was 0.017. In figures 3a and 3b all curves are

Tab. 2
Absorption differences at 240 mμ between pH 10 and pH 2 for various barbiturates at several serum* concentrations[1])

Barbiturate	Absorption differences at 240 mμ obtained for a barbiturate concentration in serum of mg/l: 50	67	100	133	150	160
Barbital		147	216	273	310	325
		143	202	274		
Butobarbital		154	208	266	302	
			188			
			214			
Cyclobarbital		137	193	240	268	289
			169	254		
			197			
Allobarbital		126	177	237	273	
		130	190		263	
Aprobarbital		130	191	241	260	
			173	236		
				245		
Pentobarbital		132	169	195	236	274
			165			
Heptabarbital		111	165	215		245
		115	183			
Phenobarbital		108	135	205	244	
		109	159		254	
Heptobarbital		80	137	152	191	
			115			
Hexabarbital	50	75	121	165		
		84				
Methylphenobarbital		78	107	137		
			110			

*) 1,1 ml Serum used in procedure.
[1]) The barbiturate concentrations are obtained by mixing serum in different rations with barbiturate solutions containing 200 mg/l. The latter are obtained by dissolving 40 mg barbiturate in 4.0 ml of 0.1 N NaOH and mixing 0.2 ml of this solution with 10 ml serum.

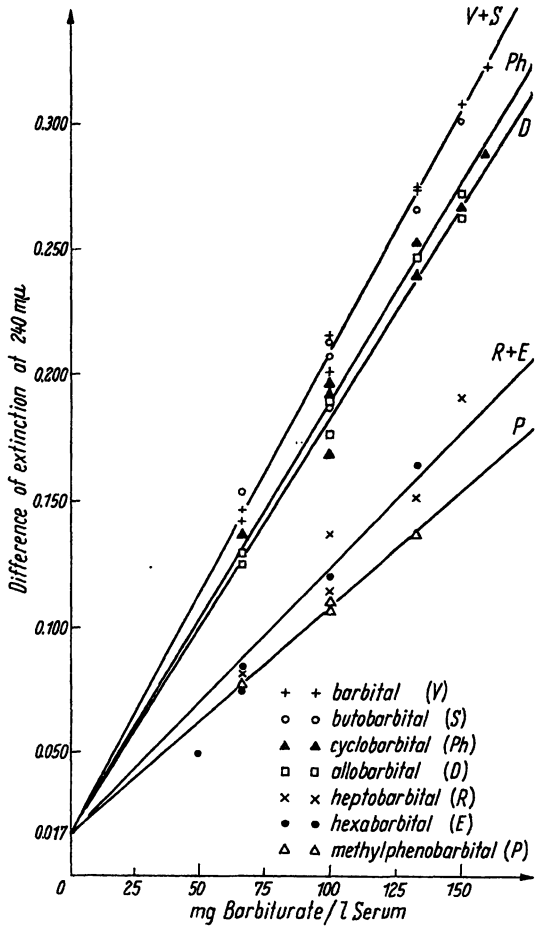

Fig. 3a
Absorption difference curves of barbiturates at 240 mμ between aqueous solutions of pH 10 and pH 2, obtained from sera containing various barbiturates of different concentrations

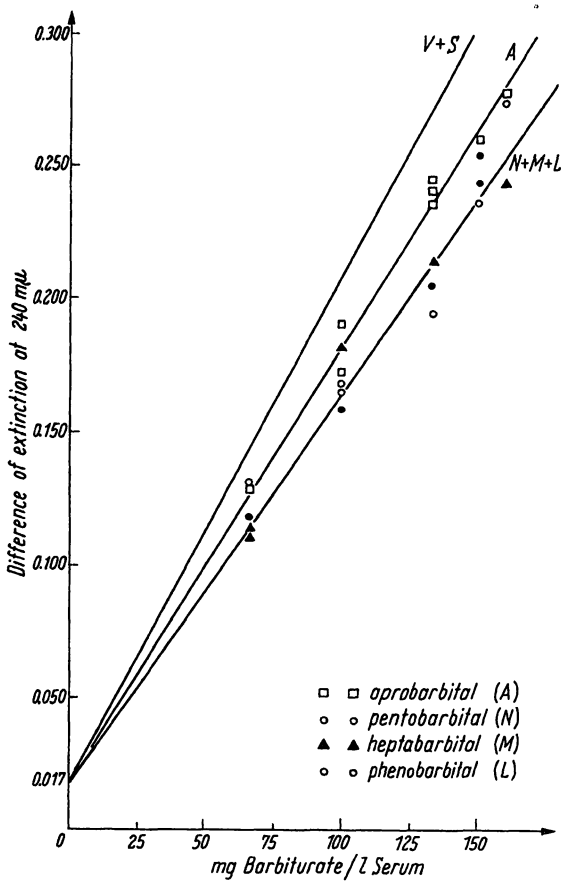

Fig. 3b
Absorption difference curves of barbiturates at 240 mμ between aqueous solutions of pH 10 and pH 2, obtained from sera containing various barbiturates of different concentrations

drawn through this point. The standard deviation of these blank values was 0.010. From figure 3 it is shown that on this basis the accuracy of the determination is not better than ± 10 mg/l serum for barbital. Low barbiturate concentrations therefore cannot be determined by this method. Since comatosis begins to appear at an "intermediate-acting" barbiturate concentration of 60 till 70 mg/l serum, the method is suitable for use in a moderate or severe case of barbiturate poisoning. Especially when the kind of barbiturate can be identified, the intoxication can be confirmed with a high degree of certainty, because it is unlikely that an interfering substance has the same parameters as a barbiturate. Acetylacetic acid also shows absorption in the barbiturate region, although there is no maximum at 240 mμ. However, in sera from diabetic patients the absorption of this substance does not seriously disturb the barbiturate identification and determination.

Conclusion

The identification of barbiturates at relative high concentrations in serum can be performed spectrophotometrically. Of various known barbiturates, the greater part can be identified with certainty, leaving two or three possible substances. A case barbiturate poisoning can be established with a high degree of certainty and its quantitative determination can be performed with more precision if the barbiturate present in serum is known.

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